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Research Article

Functional Properties of Filamentous Fungi Isolated from the Indonesian Fermented Dried Cassava, with Particular Application on Poultry

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Abstract The study aimed to evaluate the probiotic properties, antioxidant activity and fermentative capacity of *Acremonium charticola* and *Rhizopus oryzae* isolated from the Indonesian fermented dried cassava, with particular application on poultry. *A. charticola* inhibited the growth of *Escherichia coli* and *Aspergillus flavus*. *A. charticola* and *R. oryzae* grew in potato dextrose agar (PDA) adjusted to pH 3 and 8 or in PDA supplemented with bile salt up to 0.8%. After soaking for 8 hr, the survival rate of *A. charticola* in the simulated gastric juice (pH 2) and bile solutions (2% bile salt) was lower than that of *R. oryzae*. *A. charticola* and *R. oryzae* exhibited strong antioxidant activities. Compared to unfermented cassava pulp (control), the fibre content of cassava pulp tended to be lower after fermentation with *A. charticola* for 14 days. The populations of *A. charticola* and *R. oryzae* were significantly higher in fermented cassava pulp than in unfermented one. Coliform was higher in cassava pulp fermented with *R. oryzae* or *A. charticola* + *R. oryzae* compared to control after 7 days of fermentation, however, the bacteria were not different between *A. charticola*-fermented cassava pulp and control. Lactic acid bacteria (LAB) were higher in *A. charticola*- and *R. oryzae*-fermented cassava pulp than those in control, however, no difference of LAB was observed between *A. charticola* + *R. oryzae*-fermented cassava pulp and control. In conclusion, *A. charticola* exhibited antibacterial, antifungal and antioxidant activity, gastrointestinal persistence and fermentative capacity that may be beneficial for poultry industry.

Keywords *Acremonium charticola*, Fermentative capacity, Probiotic activity, *Rhizopus oryzae*

Fermentation is one of the oldest methods to preserve foods. The activity of microorganisms may protect the foods from many pathogenic and spoilage organisms, allowing the foods to stay edible longer. Apart from the preservative effect, fermentation may improve nutritional properties and elicit health benefits for human and animals [1, 2]. *Gathot* is a traditional Indonesian (Central and East Java Province) food produced by fermenting cassava spontaneously at aerobic condition. The fermentation involves several ubiquitous microorganisms including fungi. In our previous study, two species of filamentous fungi have been isolated from *gathot*, i.e., *Acremonium charticola* and *Rhizopus oryzae* [3].

Aside from mycotoxin-producing fungi, most fungi are not dangerous and some fungi indeed can exert positive effects on human and animal health [1, 4]. These beneficial effects seem to be attributed to the probiotic properties (such as antibacterial and antifungal activities and the tolerance of fungi to gastrointestinal condition) and antioxidant activity of the fungi [4]. The antibacterial and antifungal activities may eliminate the invading harmful bacteria or fungi [5], while the tolerance to gastrointestinal condition may ensure the viability and activity of fungi in the intestine of the host [4, 6]. Moreover, the antioxidant activity may be beneficial for boosting the host immune system [7]. To date, the probiotic properties and antioxidant activity of *A. charticola* and *R. oryzae* isolated from the Indonesian fermented dried cassava have not been studied.

The use of synthetic antibiotics as growth promoters and disease prevention has been practiced for more than 50 yr in poultry industry. This application may, however, have a risk to human health, e.g., phenomena antibiotic resistance in human due to antibiotic residues in food of animal origin [5]. Due to food safety reason, the use of such antibiotics for poultry production is therefore banned

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(in European Union) or reduced (in the rest of the world). It has been acknowledged that removal of synthetic antibiotics as feed additive in the ration may adversely affect the growth and health of chickens. Several alternatives have been proposed to substitute synthetic antibiotics including probiotics. Yet, the efficacy of the probiotic feed additives in poultry is inconsistent [5]. At present, lactic acid bacteria (LAB) are the most common microorganism used for probiotics. It is known that LAB are sensitive to heat, and therefore the survivability and stability of LAB as probiotics are difficult to maintain especially during feed manufacturing [8]. Note that the survivability and stability of probiotics may determine the effectiveness of probiotics in improving the performances and health of chickens [8]. Besides LAB, fungi are potential probiotic microorganisms for poultry [4]. Yudiarti *et al.* [9] reported that filamentous fungus *Chrysonilia crassa* could stimulate the development of duodenal villi and decrease the number of pathogenic bacteria and fungi in duodenum and caeca of chickens. Different from that of LAB, the ability of fungi to produce spores makes the fungi able to survive and remain stable even in the extreme condition [10]. Thus, fungal probiotics are expected capable of maintaining their survivability and stability during feed manufacturing and viability during transit through the gastrointestinal tract of the chickens.

Apart from the potential uses of fungi as probiotics, fungi are microorganisms important in the fermentation processes of feed ingredients for chickens [11]. Fungi may play an essential role in breaking down the less accessible lignocellulosic feedstuffs [12] and therefore increase the nutrient bio-availability for the animals. Fungal fermentation has also been reported to increase the protein content of feed ingredients [11]. Taken together, the fibre degrading capacity of fungi and their ability to increase protein may be beneficial for improving the quality of unconventional feed ingredients for chickens which commonly have high and low fibre and protein content, respectively. At present, the functional properties of *A. charticola* and *R. oryzae* isolated from *gathot* to lower the fibre and increase the protein content of the feed ingredients remain unelucidated. The objective of the present study was to evaluate the functional properties of filamentous fungi isolated from the Indonesian fermented dried cassava including antibacterial, antifungal and antioxidant activity, gastrointestinal persistence and fermentative capacity.

MATERIALS AND METHODS

Assay of antibacterial activity. *Escherichia coli* which is considered potentially pathogens to poultry was included in the study. Working culture of *E. coli* ATCC 25922 was prepared by retrieving the isolate from the stock culture (stored at 4°C) on plate count agar (PCA; Merck, Darmstadt, Germany). The plate was incubated at 37°C for 24 hr under aerobic condition. Concomitantly, the fungal isolates were transferred from the fungal stock culture (stored at 4°C) into potato dextrose agar (PDA; Merck) supplemented with chloramphenicol (Merck) and incubated at 37°C for 48 hr under aerobic condition. The evaluation of antibacterial activity started with punching (diameter, 6 mm) the fungus and *E. coli* growing on PDA and PCA, respectively, and growing them side-by-side on plate (diameter, 145 mm) containing PCA. The antimicrobial activity of the tested fungal isolates was determined by visually observing the zone occupied by the respective fungus in comparison to that of *E. coli* after aerobic incubation at 37°C for 24 hr. The assays were conducted in triplicates.

Assay of antifungal activity. *Aspergillus flavus* is one of the fungi causing feed spoilage and considered potentially pathogens to poultry. Hence, this fungus was included in this present study. Working cultures of *A. flavus* and the tested fungi were prepared by retrieving the isolates from the respective fungal stock cultures on PDA supplemented with chloramphenicol. The plates were incubated at 37°C for 48 hr under aerobic condition. Analyses of antifungal activity started with punching (diameter, 6 mm) the tested fungus and *A. flavus* and then growing them side-by-side on plate (diameter, 145 mm) containing PDA. The antifungal activity of the tested fungal isolates was determined as conducted for the antibacterial activity. The assays were conducted in triplicates.

Assay of antioxidant activity. Evaluation of antioxidant activity was initiated by preparing the fungal pellet from the grown culture of *A. charticola* or *R. oryzae* in potato dextrose broth after incubation at 37°C for 48 hr. The fungal pellet was obtained by centrifugation of the grown culture at 6,000 rpm for 15 min. The pellet of the respective fungus was then subjected to the 2,2-diphenylpicrylhydrazyl (DPPH) free radical scavenging assay based on Wu *et al.* [13] with few modifications. The absorbance was measured at 515 nm. Ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) which is a stable antioxidant was used as a reference, and the assays were conducted in triplicates.

Assay of tolerance to gastrointestinal conditions. Preliminary trials were conducted to evaluate the tolerance of fungi to acid and base condition as well as to bile salt. The tolerance of fungi to acid and base conditions were assessed by taking each fungal isolate from the stock culture and growing on PDA adjusted to pH 3 or pH 8, respectively. Unlike PDA at pH 8, PDA could not be solidified well at pH 3. Therefore, fungi were grown on unsolidified PDA at pH 3. The tolerance of fungi to acid and base conditions was also assessed by soaking the fungi in the solution (sterilized-distilled water) adjusted to pH 3 and pH 8 for 20 min and subsequently growing on PDA. Surviving colonies were observed following the aerobic incubation at 37°C for 48 hr and compared against the control. The tolerance of fungi to bile salt was evaluated based on agar well diffusion method. The solidified agar

was punched with a 6-mm diameter wells and filled with 25 μL of bile solution at the concentration of 0.2% (0.2 g oxgall [Difco, Detroit, MI, USA] in 100 mL sterilized-distilled water), 0.4%, 0.8%, and control (0%). The size of the inhibition zone around the wells was detected and measured following the aerobic incubation at 37°C for 48 hr. The concentrations of bile solution were prepared based on the fact that total bile salt concentrations in chicken intestine normally range from 0.01% to 0.7% [14]. The tolerance of fungi to bile salt was also assessed by soaking the fungi in the bile solutions of 0.2%, 0.4%, and 0.8% and control for 20 min. Surviving colonies were observed following the aerobic incubation at 37°C for 48 hr and compared against the control. The assays were conducted in triplicates.

In addition to the above trials, the fungal isolates were assessed for their survival on the simulated gastric juice (pH 2) and bile solution (2%) according to Lian *et al.* [15] with some modifications. The simulated gastric juice was prepared by suspending pepsin (3 g/L; Sigma-Aldrich) in saline (0.5%, v/v) and adjusting the pH to 2 with 12 N HCl. The bile solution was prepared by dissolving 2 g oxgall (Difco) in 100 mL distilled water. All solutions were then sterilized at 121°C for 15 min. Essentially, 1.0 g of the fungal pellet was suspended in a (1 : 10, wt/wt) simulated gastric juice or bile solution and was vortexed for 20 sec. The mixtures were then incubated at 37°C with manual shaking periodically (every 1 hr), and during the incubation period viable fungi were enumerated. The 1.0 mL samples taken immediately after mixing (0 hr) the fungi with gastric juice or bile solution and at the predetermined time intervals (4 and 8 hr) were suspended in a (1 : 10, wt/wt) peptone solution (Merck) and serially diluted. The viable fungi were then enumerated on PDA after aerobic incubation at 37°C for 48 hr. The assays were conducted in triplicates.

Determination of viable cell counts and proximate analysis. Cassava pulp (a by-product of cassava starch factory processing) which contains high fibre and low protein was used as a substrate of fermentation. Initially, cassava pulp was sun-dried (dry matter content *ca* 87.5%) and milled. Cassava pulp (500 g) was then put into a plastic bag, autoclaved at 121°C for 15 min and allowed to cool. The working cultures of *A. charticola* or *R. oryzae* were prepared by retrieving each fungal culture collection on PDA and incubating at 37°C for 48 hr. Fungal mycelium from the incubated plate was harvested from PDA under sterile condition by scraping with the aid of a spatula (excluding PDA), and this mycelium was diluted in 200 mL of sterilized-distilled water. The suspension (200 mL) containing either *A. charticola, R. oryzae* or *A. charticola* + *R. oryzae* was inoculated to 200 g of cassava pulp (inoculum contained *ca* 4×10^8 cfu/mL of fungi) and the mixture (cassava pulp and inoculum) was then incubated at room temperature for 14 days. The mixture was thoroughly mixed manually every 2 days and the samples (for analyses) were taken at days 7 and 14 of incubation. The sample (1.0 g) was diluted in 10 mL peptone (Merck) solution and then serially diluted with peptone solution before pour plating on the specific agar media. Colonies of *A. charticola* or *R. oryzae* and *A. flavus* were enumerated on PDA supplemented with chloramphenicol after aerobic incubation at 37°C for 48 hr. Coliform bacteria were counted on MacConkey agar (Merck) after aerobic incubation at 37°C for 24 hr. LAB were counted on de Man-Rogosa-Sharpe (Merck) agar after anaerobic incubation at 37°C for 48 hr. For the proximate analysis, the samples were dried in oven at 55°C for 24 hr before analysis. The assays were conducted in triplicates.

Statistical analysis. The data of antibacterial and antifungal activity and the data of tolerance of fungi to gastrointestinal conditions (obtained from preliminary trials) were not statistically analyzed. The data of antioxidant activity, viability of fungi after exposure to gastric juice or bile solutions, chemical composition and colonies of microorganisms growing in fermented cassava pulp were analyzed by analysis of variance (ANOVA). Duncan's *post hoc* test was used to assess differences between mean values when $p < 0.05$ [16].

RESULTS

Antibacterial activity of fungi. The antibacterial activity of the tested fungi is presented in Fig. 1. *A. charticola*

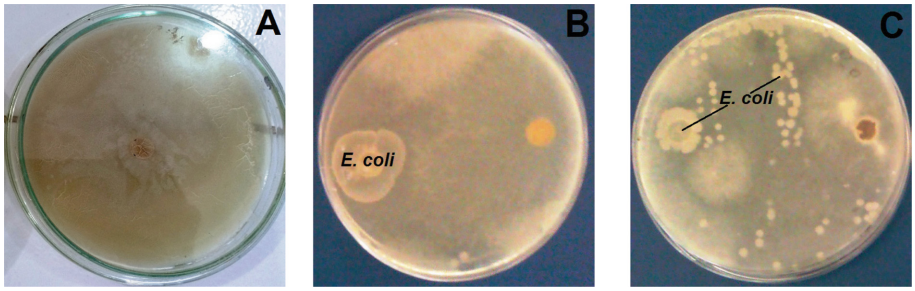

Fig. 1. The antibacterial activity of fungi against *Escherichia coli*. A, *E. coli* growing alone in plate count agar (PCA) as a control; B, *E. coli* growing together with *Acremonium charticola* in PCA (*A. charticola* occupied around 80% area of Petri dish); C, *E. coli* growing together with *Rhizopus oryzae* in PCA (*R. oryzae* occupied around 70% area of Petri dish).

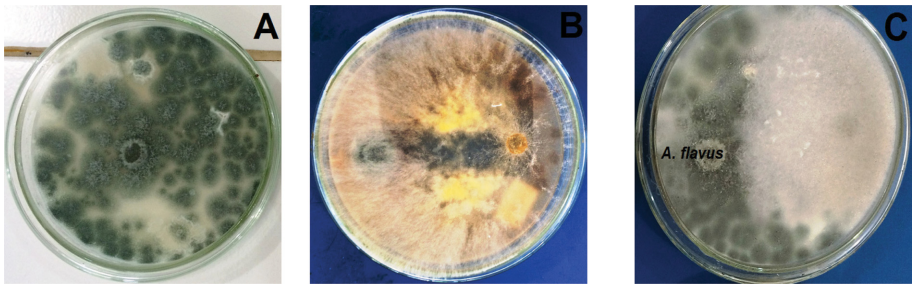

Fig. 2. The antifungal activity of fungi against *Aspergillus flavus*. A, *A. flavus* growing alone in potato dextrose agar (PDA) as a control; B, *A. flavus* growing together with *Acremonium charticola* in PDA (*A. charticola* occupied almost 100% area of Petri dish); C, *A. flavus* growing together with *Rhizopus oryzae* in PDA (*R. oryzae* occupied around 50% area of Petri dish).

Table 1. DPPH radical scavenging activity of fungal isolates[a]

Fungal isolates	IC_{50} [b] (μg/mL)
Acremonium charticola	51.96 ± 0.01 a
Rhizopus oryzae	55.89 ± 0.63 b
Ascorbic acid (standard)	1.89 ± 2.88 c

Values with different letters within the same column were significantly different ($p < 0.05$).
[a]Values are mean ± SD (n = 3).
[b]IC_{50} is identified as the effective concentration at which the 2,2-diphenylpicrylhydrazyl (DPPH) radicals were scavenged by 50%. A higher of DPPH radical scavenging activity is associated with a lower IC_{50} value.

inhibited the growth of *E. coli* in PCA. Compared to *A. charticola*, *R. oryzae* seemed to inhibit the growth of *E. coli* in a lower extent (*A. charticola* occupied around 80% area of Petri dish; *R. oryzae* occupied around 70% area of Petri dish).

Antifungal activity of fungi. The antifungal activity of the tested fungi is presented in Fig. 2. *A. charticola* could inhibit the growth of *A. flavus* in PDA. However, *R. oryzae* seemed to inhibit the growth of *E. coli* by a much lower extent as compared to *A. charticola* (*A. charticola* occupied almost 100% area of Petri dish; *R. oryzae* occupied around 50% area of Petri dish).

Antioxidant activity of fungi. Result in the present study demonstrated that both fungal isolates exhibited strong antioxidant as indicated by the values of IC_{50} below 100 μg/mL (Table 1). In general, the IC_{50} values of *A. charticola* and *R. oryzae* were higher ($p < 0.05$) than those of ascorbic acid as a synthetic standard, and the IC_{50} values of *A. charticola* were lower ($p < 0.05$) than those of *R. oryzae*.

Tolerance of fungi to gastrointestinal conditions. In the preliminary trials, both fungal isolates showed their tolerance to acid and base conditions. *A. charticola* was able to grow in both acid and base PDA, although the fungi seemed to grow better in the base than in acid PDA

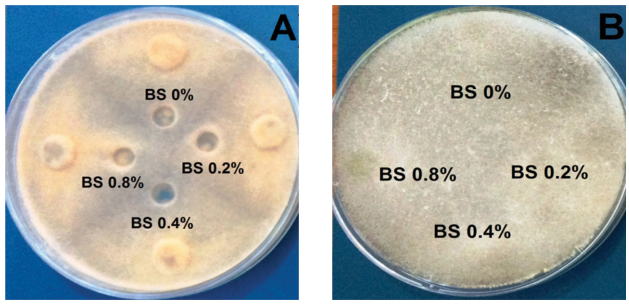

Fig. 3. The tolerance of fungal isolates to bile solution. A, *Acremonium charticola* growing in potato dextrose agar (PDA) supplemented with bile solution 0%, 0.2%, 0.4%, and 0.8%; B, *Rhizopus oryzae* growing in PDA supplemented with bile solution 0%, 0.2%, 0.4%, and 0.8%. The inhibition zones around the wells filled with the bile solutions were not detected. BS, bile salt.

(data not presented). The same condition was observed for *R. oryzae*. Moreover, the fungi were able to grow in PDA following the soaking in solutions adjusted to pH 3 or pH 8 (data not presented). In term of tolerance of fungi to bile salt, the inhibition zone around the wells filled with the bile solutions up to 0.8% were not detected in this study, and therefore the inhibition zone around the wells was not measured (Fig. 3). In addition, the fungi grew in PDA following soaking in the bile solutions up to 0.8% (data not presented).

Fig. 4 shows the time course of the survival of *A. charticola* and *R. oryzae* exposed to the simulated gastric juice (pH 2) or bile solutions for a period of 8 hr. In general, the survival of the fungal isolates decreased as the exposure time increased. For the entire time of exposure, the survival of *A. charticola* in the simulated gastric juice and bile solutions was lower ($p < 0.05$) compared to that of *R. oryzae*.

Viable cell counts and proximate analysis. Table 2 shows the colonies of microorganisms growing in cassava pulp during the fermentation. Compared to unfermented cassava pulp (control), the colonies of *A. charticola* and *R. oryzae* were higher ($p < 0.05$) in cassava pulp after

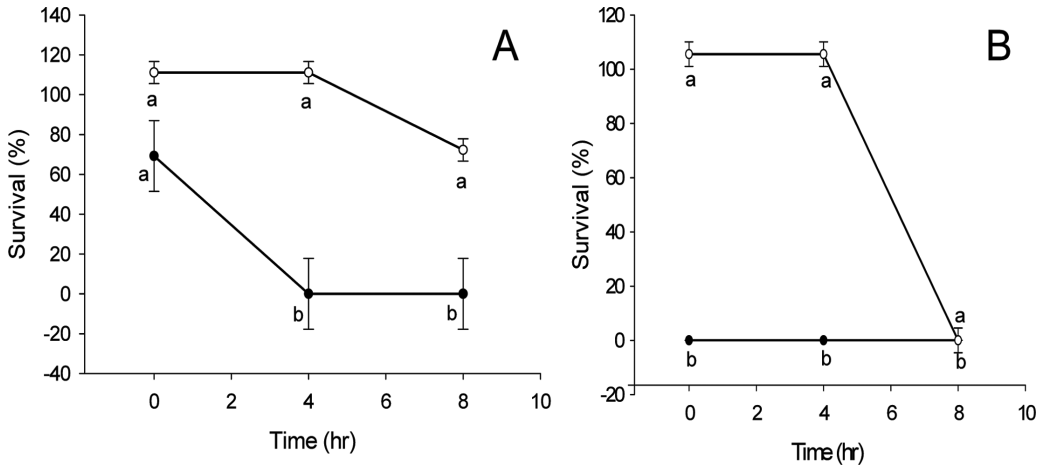

Fig. 4. Time course of the survival of *Acremonium charticola* (–●–) and *Rhizopus oryzae* (–○–). A, Viability of fungi after exposure to simulated gastric juice at pH 2; B, Viability of fungi after exposure to bile solutions 2%. Survival is presented as percentage of surviving population (cfu/mL) at different sampling times relative to initial population (cfu/mL). [a,b]Values with different letters within the same hour were significantly different ($p < 0.05$).

Table 2. Viable cell counts of the cultures of cassava pulp[a]

	Microorganisms (log cfu/g)			
	Tested fungi	*Aspergillus flavus*	Coliform	LAB
Day 7 of fermentation				
Control[b]	0.00 ± 0.00 a	0.00 ± 0.00	0.00 ± 0.00 a	0.00 ± 0.00 a
Acremonium charticola	5.75 ± 0.67 b	0.00 ± 0.00	2.87 ± 2.80 ab	4.67 ± 0.58 b
Rhizopus oryzae	5.73 ± 0.64 b	2.24 ± 3.89	3.90 ± 2.48 b	5.39 ± 0.93 b
A. charticola + *R. oryzae*	5.67 ± 0.58 b	2.06 ± 3.57	4.00 ± 1.00 b	3.00 ± 3.00 ab
Day 14 of fermentation				
Control[b]	0.00 ± 0.00 a	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00 a
A. charticola	> 10.00 ± 0.00 b	0.00 ± 0.00	1.77 ± 3.06	9.77 ± 1.57 b
R. oryzae	> 8.67 ± 1.53 b	3.49 ± 6.05	2.00 ± 3.46	9.20 ± 2.82 b
A. charticola + *R. oryzae*	> 9.66 ± 0.58 b	6.48 ± 5.87	3.67 ± 3.21	5.83 ± 5.53 ab

Values with different letters within the same column and days of fermentation were significantly different ($p < 0.05$).
The symbol ">" indicates that some observations from which the mean was calculated had values above detection levels. When the colonies could not be counted on the plates, the detection level was applied and used to make the calculations. Therefore, the real mean value is above than that reported.
[a]Values are mean ± SD (n = 3).
[b]Unfermented cassava pulp.

Table 3. Chemical compositions of fermented cassava pulp[a]

	Chemical composition (%)			
	Crude protein	Ether extract	Crude fibre	Ash
Day 7 of fermentation				
Control[b]	2.14 ± 0.23	0.78 ± 1.04	18.43 ± 0.63	4.25 ± 1.25
Acremonium charticola	2.23 ± 0.22	0.45 ± 0.16	16.92 ± 5.07	3.42 ± 0.26
Rhizopus oryzae	2.50 ± 0.14	0.42 ± 0.09	17.64 ± 4.72	3.51 ± 0.41
A. charticola + *R. oryzae*	2.44 ± 0.20	0.44 ± 0.07	17.11 ± 0.94	3.21 ± 0.06
Day 14 of fermentation				
Control[b]	2.14 ± 0.23	0.78 ± 1.04	18.43 ± 0.63	4.25 ± 1.25
A. charticola	2.17 ± 0.19	0.50 ± 0.22	14.24 ± 2.31	3.42 ± 0.30
R. oryzae	2.18 ± 0.31	1.27 ± 0.88	15.64 ± 4.47	3.93 ± 1.00
A. charticola + *R. oryzae*	2.17 ± 0.28	0.57 ± 0.33	15.31 ± 0.84	4.03 ± 1.16

[a]Chemical compositions of fermented cassava pulp are expressed as dry matter basis; values are mean ± SD (n = 3).
[b]Unfermented cassava pulp.

fermentation for 7 or 14 days. The colonies of coliform bacteria were higher ($p < 0.05$) in cassava pulp fermented with *R. oryzae* or *A. charticola* + *R. oryzae* when compared with control after 7 days of fermentation; however, the bacteria were not different between *A. charticola*-fermented cassava pulp and control. The populations of LAB were higher ($p < 0.05$) in *A. charticola*- and *R. oryzae*-fermented cassava pulp than those in control, however, no difference of LAB was observed between *A. charticola* + *R. oryzae*-fermented cassava pulp and control.

The chemical compositions of fermented cassava pulp are presented in Table 3. Compared to unfermented cassava pulp, the fibre content of cassava pulp tended ($p = 0.08$) to be lower after fermentation with *A. charticola* for 14 days. The content of protein, fat and ash did not differ between unfermented and fungal-fermented cassava pulp. There was no difference with regard to chemical compositions of cassava pulp after fermentation for 7 and 14 days.

DISCUSSION

Preservation is one of the main purposes of food/feed fermentation which carried out by various microorganisms including fungi. At this point, the ability of fungi (as an inoculum) to inhibit the growth of pathogenic and spoilage microorganisms play a crucial role. In the present study, *A. charticola* isolated from *gathot* could inhibit the growth of *E. coli* and *A. flavus in vitro*. The mechanisms by which *A. charticola* inhibited the growth of *E. coli* and *A. flavus* remain unclear, but one possible mechanism could be that the fungus produced some form of antimicrobials and antifungals that may impair the biological functions of microorganisms [17]. In the present study, both fungi isolated from *gathot* showed strong antioxidant activities as indicated by the low IC_{50} values. According to Blois [18], samples with $IC_{50} < 50$ μg/mL have very strong antioxidant, 50~100 μg/mL strong antioxidant, 101~150 μg/mL medium antioxidant and > 150 μg/mL weak antioxidant. In term of food/feed storage, this property is beneficial to protect the food/feed from oxidative degradation by free radicals and therefore prolong the storage of food/feed [13]. From this consideration, *A. charticola* and *R. oryzae* may be valuable natural antioxidant source that can potentially be applicable in poultry feed industry.

In addition to the food preservative potential, some fungi have been reported to possess probiotic properties which are beneficial for human and animal health [1, 4]. In general, the antimicrobial and antifungal activities are of essential properties of probiotic, which may inhibit the growth of pathogenic bacteria and fungi invading the body [4, 5]. Unlike *R. oryzae*, *A. charticola* inhibited the growth of *E. coli* and *A. flavus in vitro*. Hence, *A. charticola* could be a good candidate of probiotic for poultry. It has widely been known that antioxidants are associated with the healthy immune system. Antioxidants may protect the immune cells from environmental damage and seem to be essential for an optimal function of the immune system [7]. The strong antioxidant activity of *A. charticola* therefore further supported the probiotic potential of this fungus in improving the host immune systems. In this present study, we did not perform *in vivo* chicken experiment. Hence, further animal experiments of poultry are worthy to verify the probiotic potential and antioxidant activity of *A. charticola* in poultry.

To function as probiotic (in the intestine), fungi need to survive transit through the stomach and colonize the intestine of the host [7]. In our preliminary study, fungi were evaluated for their tolerance to acid (pH 3) and base (pH 8) conditions as well as to different concentrations of bile salt. Both fungal isolates were able to survive in acid and base conditions as well as in solutions containing bile salt up to 0.8%. In the later experiment, the probiotic potential of the fungal isolates was further evaluated based on their ability to survive in simulated gastric juice (pH 2) and in higher concentration of bile solution (2%) for 8 hr. Throughout the exposure time, the survival rate of *R. oryzae* in simulated gastric juice and bile solutions was higher when compared to *A. charticola*. The different sensitivity of the fungal spores to acid/simulated gastric juice and bile solution seemed to influence the survival rate of the tested fungi, as reported by Duc *et al.* [19] in *Bacillus* species and Ali *et al.* [20] in *Saprolegnia*. These latter results may raise the question if it is worth using *A. charticola* as probiotic especially for chickens. However, the fact that the pH of chicken stomach is 3.54 [21] may reveal the importance of *A. charticola* as a potent probiotic for chickens as this fungus was able to survive in the medium adjusted to pH 3. Correspondingly, the ability of *A. charticola* to grow in the medium containing bile salt up to 0.8% may further reveal the possibility of *A. charticola* as a probiotic for chickens given that the total bile salt concentrations in chicken intestine range from 0.085 to 7.00 mg/mL or 0.0085% to 0.7% [14].

In commercial poultry production, feed is considered the most important input and plays a major role in total production cost. Due to global rise in the price of feed ingredients, there is now a tendency in the poultry industry to move toward the use of unconventional feed ingredients such as cassava pulp that is a by-product of cassava starch factory processing. This, however, is limited by the high fibre content in cassava pulp that can reduce its digestibility. Earlier study have shown that fungal fermentation decreased the fibre content and increased the crude protein content of cassava pulp [11]. In the present study, fermentation with *A. charticola* for 14 days could decrease the fibre content of cassava pulp. However, fungal fermentation for 7 or 14 days did not increase the protein content of cassava pulp. The latter result may indicate that *A. charticola* and *R. oryzae* could not use the free nitrogen from atmosphere to synthesize protein and/or the production of fungal biomass protein was limited during fermentation. To increase the protein content of fermented cassava pulp, it seems therefore

necessary to enrich the cassava pulp with nitrogen source, for instance urea, during fermentation as conducted by Khempaka *et al.* [11] when fermenting cassava pulp with *Aspergillus oryzae*. Owing to this, further fermentations had subsequently been conducted by inoculating cassava pulp with fungal starter (*ca* 4×10^8 cfu/mL) and 41 g/kg urea (on dry matter basis). After incubation for 14 days, the protein content of cassava pulp increased from 2.14 ± 0.23% to 11.34 ± 0.35%, 12.80 ± 0.55% and 14.77 ± 0.25% for *A. charticola*-, *R. oryzae*-, and *A. charticola* + *R. oryzae*-fermented cassava pulp.

The fermented feed is attributable to the high content of particular microorganisms used as inoculation starter. In this study, fermentation resulted in higher populations of *A. charticola* and/or *R. oryzae* in cassava pulp when compared with unfermented cassava pulp. In addition, the populations of the inoculation starters (*A. charticola* and *R. oryzae*) were doubled from day 7 to day 14 of fermentation. Based on this result, it is necessary to ferment the cassava pulp with *A. charticola* and/or *R. oryzae* for 14 days in order to produce fermented cassava pulp with probiotic properties. Note that to exert the beneficial effects; fermented feed must contain probiotic microorganisms above 10^6 cfu/g or mL [22]. It has been reported that fungal fermentation resulted in decreased pH value of the substrates [23]. This low pH may favour the growth of LAB [2] that is beneficial for the health and performances of chickens [5]. In accordance with this, our present results showed that fermentation of cassava pulp with *A. charticola* or *R. oryzae* led to higher populations of LAB (though the pH was not measured) when compared to control. It has commonly been known that the reduction in pH and the increase in LAB population can inhibit the growth of pathogenic organisms such as coliform bacteria from developing in the feed [2]. Concomitantly, the higher LAB content (and possibly the lower pH) in the current fermented cassava pulp was associated with the lower population of coliform bacteria.

In conclusion, fungi isolated from the Indonesian fermented dried cassava, particularly *A. charticola*, exhibited antibacterial, antifungal and antioxidant activity, gastrointestinal persistence and fermentative capacity that may be beneficial for poultry industry. Further in vivo chicken study is needed to evaluate the probiotic activity of *A. charticola* on the health and performances of chickens.

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